Steroidal Dimer by001 Inhibits Proliferation and Migration of Esophageal Cancer Cells via Multiple Mechanisms

Sai-Qi Wang 1,4, Kai-Rui Zhou 1,4, Xiao-Li Shi, Li-Ming Chang, Yan-Bing Zhang, Bin Yu*, Hong-Min Liu*

School of Pharmaceutical Sciences & Collaborative Innovation Center of New Drug Research and Safety Evaluation, Key Laboratory of Henan Province for Drug Quality and Evaluation, Zhengzhou University, Zhengzhou 450001, China

Abstract: Following our previous success in identifying new steroid-based anticancer agents, we herein disclosed the structural requirements for retaining high potency against cancer cells and associated modes of action. The structurally novel steroidal dimer by001 inhibited growth of different esophageal cancer cells and colony formation at low micromolar levels, elevated cellular ROS levels and caused mitochondrial dysfunction. Mechanistic studies showed that by001 induced cell death through the mitochondria and death receptor-mediated apoptotic pathways and autophagy induction, as well as inhibited migration.

Keywords: Steroidal dimer; Steroidal N-heterocycles; Antiproliferative activity; Esophageal cancer cells

1. Introduction

Steroids, an important class of polycyclic compounds, are essential in maintaining normal physiological functions and also play important roles in the treatment of diverse diseases. Due to the low abundance and synthetic intractability of natural steroids, many efforts have been devoted to chemical modifications of commercially available steroids, generating millions of structurally novel steroidal compounds (particularly the steroidal N-heterocycles) with interesting and diverse biological activities [1-5]; some of these steroidal derivatives have advanced into clinical trials or are currently being used for the treatment of diseases. For examples, abiraterone [6] and galeterone [7,8] derived from dehydroepiandrosterone (DHEA) have been used in clinic for the treatment of all-stage prostate cancers. Among steroids, of particular interest are the steroidal dimers, which are also found in nature (e.g. cephalostatin I, ritterazines A, and crellastatin A) and show potent antiproliferative activities against human cancer cell lines [9,10]. Inspired by the structural novelty and promising bioactivities of steroidal dimers, a large number of new steroidal dimers have been synthesized and assessed for their biological potential [11-13]. However, their detailed mechanisms of action are less studied. In continuation with our previous interest in identifying potent anticancer agents, we screened our in-house steroid-based molecular library using the MTT assay [14-24], leading to the identification of a structurally symmetric [1,2,4] triazolo [1,5-a] pyrimidine-based steroidal dimer (named by001) having potent antiproliferative activity against several human cancer cell lines, remarkably more potent than its analogs previously synthesized in our group (Fig. 1). Herein, we disclose its mechanisms of inducing cell death and the ability of inhibiting migration. by001 may serve as a template for designing potent steroid-based anticancer agents. Further work will be focused on investigating the structure-activity relationships (SARs) through the structural simplification strategy considering the high molecular weight (about 803) of by001.
2. Results and discussion

2.1. Evaluation of antiproliferative activities and SARs studies

With by001 in hand, we next evaluated the antiproliferative activities against human cancer cells of different origins including esophageal (EC109 and TE-1), gastric (MGC-803), neuroblastoma (SH-SY5Y), prostate (PC-3), hepatocarcinoma (SMMC-7721) cancers using the MTT assay. DHEA and 5-FU were used as the control. The gastric epithelial cell line GES-1 was used to study the selectivity between the cancer cells and normal cells, as well as the potential toxicity. As shown in Fig. 2, the cell viability of several cancer cells decreased significantly in a concentration-dependent manner after treated with by001 for 72 h, while cells treated with DHEA and 5-FU showed a gradual decline in cell viability. The IC50 values of by001 against different cancer cells and human gastric epithelial cell line GES-1 are listed in Table 1. Evidently, except for SMMC-7721 cells (IC50 = 16.11 µM), by001 showed good inhibition against the tested cancer cell lines (IC50 < 2.8 µM), especially against SH-SY5Y and PC-3 cells (IC50 = 0.41 and 0.76 µM, respectively). In contrast, ZFY001 (Fig. 1) displayed weak inhibitory activity against PC-3 cells, highlighting the importance of dimer scaffold. However, we can also observe that by001 also inhibited growth of normal GES-1 cells potently with an IC50 value of 2.76 µM, similar to that of MGC-803 cells (IC50 = 1.92 µM), suggesting the low selectivity of by001. Collectively, by001 is a structurally new cytotoxic agent, highly potent than DHEA and ZYF001. These data suggest that the [1,2,4] triazolo [1,5-a] pyrimidine scaffold and the dimer skeleton are necessary for the activity.
Figure 2. Cell viability after treatment with by001 for 72 h. Data were shown as mean ± S. D. from three independent experiments.

Table 1. IC₅₀ values of by001, DHEA, and 5-FU against human cancer cell lines and GES-1

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>by001 (μM)</th>
<th>DHEA (μM)</th>
<th>5-FU (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC109</td>
<td>1.90 ± 0.73</td>
<td>24.66±3.65</td>
<td>4.61±0.53</td>
</tr>
<tr>
<td>TE-1</td>
<td>2.71 ± 0.38</td>
<td>87.24±9.12</td>
<td>15.10±2.54</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>0.41 ± 0.12</td>
<td>67.42±2.62</td>
<td>6.93±0.46</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.76 ± 0.16</td>
<td>20.36±1.37</td>
<td>7.87±1.31</td>
</tr>
<tr>
<td>SMMC-7721</td>
<td>16.11 ± 1.27</td>
<td>104.98±2.36</td>
<td>17.12±1.00</td>
</tr>
<tr>
<td>MGC803</td>
<td>1.92 ± 0.38</td>
<td>17.51±1.58</td>
<td>13.17±1.64</td>
</tr>
<tr>
<td>GES-1</td>
<td>2.76 ± 0.46</td>
<td>&gt;150</td>
<td>7.95±1.77</td>
</tr>
</tbody>
</table>

2.2. By001 reduced colony formation of EC109 cells

Colony formation assay is a common in vitro cell survival assay to test the ability of a single cell growing into a colony. To study the effect of by001 on the colony formation in EC109 cells, different concentration of by001 was used to treat the cells for 7 days. In Fig. 3A, by001 concentration-dependently reduced the number of colony of EC109 cells, and by001 at 3.0 μM inhibited nearly 95% of colony formation. Treatment of by001 also caused a sharp decrease of the size of the colonies (Fig. 3B). The results indicate that by001 is capable of inhibiting the colony formation of EC109 cells.
In our previous study, we found that by001 decreased the mitochondrial membrane potential (MMP) and induced apoptosis of EC109 cells [14]. However, the exact mechanism of inducing cell death remains unclear. To study the underlying mechanisms, we measured the MMP of EC109 and TE-1 cells using the JC-1 dye. The intensity of green fluorescence reflects the loss of mitochondrial membrane potential. As shown in Fig. 4, after treatment with by001 for 24 h, the green fluorescence intensity in EC109 and TE-1 cells increased with the increasing concentration of by001, accompanied with decrease of red fluorescence intensity, indicating the loss of MMP. The results suggest that by001 caused mitochondrial dysfunction, thereby inducing apoptosis in esophageal carcinoma cells EC109 and TE-1.
2.3. By001 triggered cellular ROS generation

Compared to normal cells, cancer cells have higher demand on the mitochondrial respiratory chain to generate more ATP for their rapid growth and differentiation, thus inevitably making cancer cells have high ROS levels. To determine whether by001 triggers ROS generation in esophageal cancer cells, the fluorescent probe 2’,7’-dichlorodihydrofluorescin diacetate (DCFH-DA) was used to detect the ROS levels in TE-1 and EC109 cells after treatment with by001. DCFH-DA is diffused into cells and then enzymatically hydrolyzed by cellular esterases to give the non-fluorescent 2’,7’-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized by ROS to form highly fluorescent 2’,7’-dichlorodihydrofluorescein (DCF). After treatment with by001 for 24 h, the fluorescence of DCF was measured by the high-content screening. As shown in Fig. 5, by001 concentration-dependently increased the mean fluorescence intensity of DCF in EC109 and TE-1 cells. These results indicate that by001 may elevate cellular ROS levels, thereby triggering apoptosis of EC109 and TE-1 cells."
2.4. Mechanistic studies of by001 inducing apoptosis of esophageal cancer cells

Based on the observed potent anticancer efficacy of by001, we first examined the expression changes of apoptosis-related proteins in EC109 cells. As shown in Fig. 6, for the Bcl-2 family proteins, the expression levels of pro-apoptotic proteins Bax and Bak were upregulated with the increasing concentration of by001, and the anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 were correspondingly down-regulated. Besides, treatment of EC109 cells with by001 also increased the expression levels of Fas, cleaved caspase-8, and caspase-9, which then activated caspase-3 and cleaved PARP, finally leading to the DNA damage and apoptosis. Interestingly, we also found that by001 also led to the cleavage of LC3I, forming LC3II, which is involved in the induction of the autophagy. Taken together, the results suggest that by001 may induce the death of EC109 cells via triggering autophagy and apoptosis through both mitochondrial and death receptor pathways (Fig. 6). Treatment of another esophageal cancer cell line TE-1 with by001 also led to similar results (Fig. 7). The expression levels of Bax were almost upregulated, while the Bcl-2 was down-regulated. By001 also increased expression levels of Fas, activated caspase-8, caspase-9 and then caspase-3, finally leading to apoptosis of TE-1 cells.
2.5. By001 inhibited migration of EC109 cancer cells

The tumor development is usually accompanied with neoplasm metastasis, which would lead to the failure of therapy, neoplasm recurrence, and even death. Herein, we determined the ability of by001 affecting migration of esophageal cancer cell EC109 using the transwell assay. EC109 cells were seeded in the upper chamber and treated with by001 for 24 h. As shown in Fig. 8, by001
concentration-dependently suppressed migration of EC109 cells, the migration rates at 0.25, 1.0 and 3.0 µM were about 53%, 70% and 86%, respectively.

Figure 8. By001 suppressed migration of EC109 cells. EC109 cells were suspended in medium with different concentrations of by001 and seeded in the upper chamber. After incubation for 24 h, the cells that migrated to the lower chamber were stained by Hoechst 33342 and detected using the high content screening system. ** P < 0.01.

Based on above results, we can conclude that by001 inhibited colony formation, elevated cellular ROS levels, and then caused mitochondrial dysfunction. By001 induced cell death via triggering autophagy and inducing apoptosis through both the mitochondrion and death receptor-mediated pathways, as well as inhibited migration of esophageal cancer cells (Fig. 9).

Figure 9. Schematic illustration of by001 inducing death of EC109 cells

3. Materials and Methods

3.1 MTT assay

Cells were seeded into the wells of the flat-bottomed 96-well culture plates in RPMI-1640 medium at a density of 3000-5000 cells per well. After an overnight attachment period, the cells were treated with medium containing 0, 0.5, 1, 2, 4, 8, 16, 32 µM of by001. After incubation for 72 h, 20 µL MTT (5 g/L) was added into each well, and incubation was continued for 4 h at 37°C. Then, supernatant was removed; formazan was solubilized from cells by 150 µL DMSO. Absorbance values were measured at 570 nm after shaking for 10 min. IC50 values were calculated using SPSS 20.0 software.
3.2 Colony formation assay

Cells were seeded into 6-well plate with a density of 1000 cells per well. After treatment of by001 (0, 0.25, 1, 3 µM), cells were cultured for 7 days. The colonies were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. The colonies containing 50 or more cells were numbered by Image J software. The area of a single clone was measured by Nikon NIS analysis software.

3.3 Detection of mitochondrial membrane potential

Cells were treated by by001 (0, 5, 10, 20 µM) for 24 h before stained with JC-1 (5 µg/mL) at 37 °C for 20 min and then washed with warmed medium without fetal bovine serum. The stained cells were taken photos and analyzed with laser scanning microscope (Nikon A1, Tokyo, Japan). Quantitative analysis was performed using the NIS-Elements AR software (Nikon).

3.4 ROS detection

The intracellular ROS was measured with DCFH-DA. Cells were cultured in 6-well plates under various conditions (0, 5, 10, 20 µM) for 6 h. Then the cells stained with DCFH-DA (10µM) for 20 min, washed with PBS three times. The fluorescence of DCFH was detected by high content screening system (Array Scan XTI, Thermo Fisher, Waltham, MA).

3.5 Western blot

The Western Blot was performed as we previously described. [25] The collected cells were lysed on ice RIPA buffer and the protein concentrations were determined using the BCA Protein Assay Kit. Samples were separated by SDS-PAGE on a 10% polyacrylamide gel and then transferred onto a PVDF membrane. The transferred membranes were blocked for 1 h in 10% nonfat milk in TBST (TBS containing 0.05% Tween-20) and incubated with primary antibodies overnight at 4°C. The goat anti-rabbit secondary antibody incubated for 2 h at room temperature. The protein bands were visualized using the enhanced chemiluminescence (ECL) Western Detection System. Relative protein levels were quantified by Image J software. β-actin was used as the loading control.

3.6 Migration assay

Cells were seeded in the in the upper chamber and cultured in the medium with 2% fetal bovine serum and by001 (0.25, 1, 3 µM) for 24 h. Then, the cells that migrated to the lower chamber were fixed with 4% paraformaldehyde and stained by Hoechst 33342 and numbered using the high content screening system (Array Scan XTI, Thermo Fisher, Waltham, MA).

4. Conclusions

Inspired by the anticancer potential of steroidal N-heterocycles and following our previous success in identifying steroid-based anticancer agents, we described the anti-proliferative activity of a structurally novel steroidal dimer by001 and its mechanisms of inducing death of esophageal cancer cells. By001 inhibited the growth of different cancer cells at low micromolar levels and colony formation, elevated cellular ROS levels and caused mitochondrial dysfunction. Mechanistic studies showed that by001 induced cell death through the mitochondrial and death receptor-mediated apoptotic pathways and autophagy induction, as well as inhibited migration. Although by001 also inhibited growth of normal GES-1 at low micromolar levels, it may serve as a template for designing potent steroid-based anticancer agents due to its excellent anticancer potency. Our results may offer an evidence that dimeric steroidal scaffolds have improved biological activities relative to the corresponding monomers.

Acknowledgments: We are grateful for the financial support from the Natural Science Foundation of China (No. 21372206, 81673322), the Development Foundation of Priority, Ministry of Education (No. 20134101130001), and National Key Research Programs of Proteins (2016YFA0501800), and the Starting Grant of Zhengzhou University (32210533).
Author Contributions: Sai-Qi Wang and Bin Yu designed the experiments; Sai-Qi Wang, Kai-Rui Zhou, Xiao-Li Shi and Li-Ming Chang performed the experiments; Sai-Qi Wang, Yan-Bing Zhang and Hong-Min Liu analyzed the data; Sai-Qi Wang and Bin Yu wrote the paper.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

References


